ALKALI-DISSOLVED DIPHTHERIA TOXOID-ANTITOXIN FLOCCULES ADSORBED ON ALUMINIUM CARRIERS

PREPARATION, AND IMMUNITY EXPERIMENTS IN ANIMALS

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The three diphtheria antigens commonly used to immunize man against diphtheria are formol-toxoid, alum-precipitated formol-toxoid (A.P.T.), and toxoid-antitoxin floccules (T.A.F.). A.P.T. has been the antigen of choice for babies and young children, in whom it produces a satisfactory immunity, after two spaced injections, with a minimum of untoward reactions of either a local or a general nature. Unfortunately, with increasing age, man becomes increasingly sensitive to the protein of *Corynebacterium diphtheriae* and, of the three prophylactics, A.P.T. is the one that is most likely to cause reactions in people over the age of 6 years. Thus, in older children, adolescents, and adults, recourse is had to formol-toxoid or to T.A.F. Formol-toxoid, although less liable to cause reactions than A.P.T., is still not free from this disadvantage, and, in addition, is to be relied upon to produce a good immunity only after three spaced injections. T.A.F. is undoubtedly the most easily tolerated of the three antigens, but, like formol-toxoid, produces a satisfactory immunity only after three injections. Further, T.A.F., in terms of freedom from bacterial protein, is undoubtedly the purest of the three antigens. It is assumed that floccules contain pure toxoid and pure antitoxin plus, at most, a very small amount of bacterial protein adsorbed by, and carried down with, the floccules. While it is appreciated that the 'purity' of the toxoid moiety is high, it is realized that the overall 'purity' is low, because the bulk of the floccules is made up of antitoxin.

Crude formol-toxoid may be freed from most of its bacterial and broth protein by selective precipitation and adsorption methods, and purities of the order of 0·0005–0·002 mg. N/Lf obtained. In terms of pure toxoid, which contains between 0·0005 mg. N/Lf (Eaton, 1936) and 0·00046 mg. N/Lf (Pappenheimer, 1937), such toxoids are pure or almost pure to 75 % impure. There is no practical advantage gained in issuing a purified product unless it produces at least as good an immunity as the raw toxoid, with fewer and less severe reactions. Further, it cannot be assumed that a toxoid of low nitrogen content will, of necessity, cause no, or only a minimal, reaction in an adult. The purification process may eliminate all, or nearly all, the broth protein and nitrogenous matter of small molecular size but leave sufficient bacterial protein to cause a reaction. Finally, even an absolutely pure toxoid could cause reactions in those individuals who are sensitive, not to bacterial protein, but to toxoid itself (Pappenheimer & Lawrence, 1948).

Work* carried out with *Clostridium welchii*, Type A, T.A.F. had shown that the floccules, dissolved in N/20 NaOH, left for 1 hr. at between 23° and 27° C. and then readjusted to pH 7 contained titratable flocculable antigen, with an overall recovery of about 50%. Such dissolved floccules, precipitated with alum, formed an excellent antigen when tested in guinea-pigs. On the not unreasonable assumption that T.A.F. would most easily yield toxoid in its purest state, in terms of its freedom from bacterical protein, this method was applied to diphtheria floccules.

Preparation of alum-precipitated dissolved floccules from toxoid

A fully detoxicated toxoid should be freed from particulate matter by paper filtration. A flocculation test, using a quickly flocculating, refined (pepsin digested) antitoxin and 1 ml. amounts of toxoid, is carried out and the 'initial' tube noted. The amount of antitoxin to be added to the bulk of toxoid is calculated from that in the 'initial' tube. The flask containing the mixture is immersed for about one-third of its height in water at 45° C. and left there with an occasional shake until flocculation has taken place. Thereafter it is placed in the cold room until the following day, to allow the floccules to settle. The supernatant fluid is discarded, and the floccule deposit washed, either in a large horizontal centrifuge or by decantation, with changes of saline. Probably a Laval type of centrifuge would be suitable for washing purposes, but a Sharples type, with both diphtheria and *welchii* floccules, caused very considerable destruction of antigen.

The washed floccules are taken up in a volume of saline, one-tenth of that of the parent toxoid and 0·5 ml. 10/N NaOH per 100 ml. added. The NaOH should be added drop by drop with constant shaking. Complete solution of the floccules occurs. After 1 hr. at about 25° C., the pH is carefully adjusted to 7 with HCl. At this stage the Lf of the solution should be determined, and for this purpose, it should be diluted 1:5 with saline, otherwise difficulty will be experienced in detecting the mixture that flocculates first.

The dissolved floccule solution is diluted with saline to contain about 60 Lf/ml. and is sterilized by filtration through a candle (British Berkefeld, and Mandler varieties have been used with success) but not through a pad of the Seitz type, which reduces the value very considerably. When tests have proved the filtrate sterile, alum-precipitation is carried out. Enough sterile 10% potash-alum solution is added to give a 1% concentration. The pH is now between 3 and 4 and no precipitation occurs until it is raised to 5 or higher. Because the toxoid in the floccule solution is relatively free of impurities, and because such toxoid withstands a low pH badly, the pH is raised quickly to pH 5·5 or higher. A copious white precipitate, some of which is probably $Al(OH)_3$, is formed and settles quickly. The precipitate is washed three times in large amounts of sterile saline and, before the final suspension is made, a sample is removed, dissolved by the addition of sodium citrate and tested for Lf value. Finally, enough saline, plus 1:10,000 merthiolate, is added so that the value will be between 50 and 60 Lf/ml. The dissolved alum-precipitate usually flocculates more slowly than the parent toxoid

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and, occasionally, blending with a known-value toxoid that flocculates in about 15–30 min. is necessary to get a result. However, the Kf of both the dissolved floccules and of the dissolved alum-precipitate is dependent, to a large extent, on the Kf of the original crude toxoid. The smaller the Kf of this, the smaller is that of the final product. When tests show that the product, precipitated dissolved floccules (P.D.F.), is sterile it is ready for use.

Preparation of aluminium phosphate adsorbed dissolved floccules (A.D.F.)

The procedure is the same as that noted for the preparation of P.D.F. up to the point where concentrated dissolved floccules are obtained. Of this material, of between 300 and 700 Lf/ml., enough is added to aluminium phosphate suspension of pH 5·7, to give about 50 Lf/ml. It is allowed to stand for at least 1 month, preferably longer, before being used.

A number of technical points may now be discussed in some detail.

Antitoxin. Except where otherwise stated, pepsin-digested antitoxin, obtained from horse serum, has been used because it flocculates more quickly than unrefined antitoxic serum and because, being a purified product, it could be assumed that it would be less likely to contribute impurities to the floccules than raw antiserum. The values of the different batches used varied between 3000 and 6000 units/ml., as determined by the flocculation test.

Toxoid. For maximum yield in Lf/ml. and for maximum purity as expressed by mg. N/Lf, a quickly flocculating toxoid, i.e. one that flocculates in 10 l. quantities in from 10 to 20 min. should be chosen. If maximum yield is not important, a more slowly and less completely flocculating toxoid gives satisfactory results with a recovery of about 50 %. There is no conclusive evidence that a toxoid flocculating in 45–60 min. yields a less pure product than one flocculating in 10–15 min., but it is definite that one that takes several hours with constant stirring gives a poor return with decreased purity. This is illustrated by the experiment, summarized in Table 1.

Table 1. *Flocculation time, recovery and purity*

Toxoid	Lf	Kf (min.)	Lf recovery (%)	Purity (mg. N/Lf)
524	80	20*	67	0·00176
525	40	300†	28	0·006

* 10 l. flask one-third immersed in warm water.

† 10 l. stirred mechanically for the whole period while in warm water.

Amount of NaOH. Most of the preliminary work on this point was done with *Cl. welchii* toxin- or toxoid-antitoxin floccules, and the optimum conditions were those described on p. 419. This held for diphtheria floccules also, although detailed experiments were not undertaken. There was little or no difference in the Lf when the time of contact was 30 or 60 min., but the Kf was larger at 60 min. When there was a slight difference in recovery it was in favour of the 60 min. interval. The yield after 5 min. was 30–40 % lower and, after 15 min., 20 % lower than after 60 min. Immediate neutralization of the alkaline-dissolved floccules with HCl usually allowed of re-flocculation, 1 hr. at 37° C. resulted in undue destruction of toxoid

and 18 hr. at 5° C. in too little destruction of antitoxin. In actual practice, the alkali is allowed to act for 50 min. before neutralization with HCl is begun, because it takes between 5 and 10 min. to adjust the pH to 7 with accuracy.

Toxoid and antitoxin used, recovery in Lf units and purity

Much of the work was done before the importance of using a quickly and fully flocculating toxoid and of the part played by a particular antitoxin was appreciated. Three media were used, Martin's broth, Linggood's and Fenton's (1947) medium and papain digest of horse flesh (Linggood & Fenton, 1947). Table 2 summarizes the results. As shown, the recovery varied from 30 to 67 % and the nitrogen content from 0·00176 to 0·003 mg. N/Lf with a mean value of 0·00243 mg. N/Lf.

Table 2. *Recovery and purity of toxoid in dissolved floccules* (D.F.)

L. = Linggood's medium; M. = Martin's broth; P. = papain digest of horse flesh.

	Toxoid			D.F.		
Medium	Batch	Lf	mg. N/Lf	mg. N/Lf	Purification factor	Recovery (%)
L.	494	56	0·06	0·003	20	50
L.	520	56	0·079	0·0026	30	54
L.	523	48	0·071	0·0028	25	55
L.	524	80	0·035	0·00176	20	67
M.	438	52	0·041	0·0029	14	42
M.	465	36	0·097	0·002	48	53
M.	477	40	0·088	0·0022	40	53
P.	525	40	0·066	0·0022	30	30

Most of the work was carried out with one refined antitoxin, chiefly because a supply of it was available and not because it had any obvious advantages. In the experiments recorded in Table 3, the purity in terms of mg. N/Lf are recorded using one antitoxin and four toxoids, and one toxoid and four antitoxins. The nitrogen content of the dissolved floccules was between 0·002 and 0·0028 mg. N/Lf and the purification was between 15 and 21 times.

Table 3. *Purities using different toxoids and antitoxins*

Toxoid batch	Antitoxin	Toxoid (mg. N/Lf)	D.F. (mg. N/Lf)	Purification factor
428	1578	0·036	0·0024	15
434	1578	0·0414	0·0028	15
438	1578	0·0412	0·00218	19
425	1578	0·0428	0·002	21
425	1547	0·0428	0·0021	20
425	1628	0·0428	0·00228	19
425	1639	0·0428	0·0022	19

The loss of antigen could be ascribed to destruction during the alkali treatment and to solution and/or elution in the washing of the floccules. On many occasions the saline washings were noticed to be opalescent, and an attempt was made to control this source of loss. Phosphate buffer, pH 5·7 of M/15 strength, was used

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for the first two washings and of M/150 strength, pH 5·7, for the third washing. By this procedure, using two different toxoids and two different antitoxins, the percentage recoveries were 75, 61, 66, and 66. The first washing in each case was very slightly opalescent and the subsequent washings water-clear or almost so.

Most of the work on toxoid recovery had been carried out with one particular lot of antitoxin (D1) with which the recoveries were between 30 and 67% (see Table 2). The experiments summarized in the last paragraph indicated that a considerable loss occurred when saline instead of buffer at pH 5·7 was used for floccule-washing purposes. However, it was found that, with another antitoxin (D2), even if saline was used for washing, the recovery was higher than that obtained with D1. Further, when this new antitoxin was used with other toxoids, the recoveries were about the same whether saline or buffer at pH 5·7 was used.

In another experiment, floccules were prepared from one toxoid, batch 547, of 80 Lf/ml., with eight different batches of antitoxin including D1 and D2. The results of these three sets of experiments are summarized in Table 4.

Table 4. *Recovery (Lf) and purity (mg. N/Lf) of toxoids using different antitoxins and saline or buffer*, pH 5·7, *as washing fluids*

Antitoxin	Toxoid	Lf	Recovery (%)	Purity (mg. N/Lf)	Washing fluid
D1	539	64	69	0·0022	Saline
D2	539	64	84	0·0024	Saline
D2	534	76	82	0·0021	Saline
D2	534	76	79	0·002	Buffer
D2	535	72	85	0·002	Saline
D2	535	72	85	0·0022	Buffer
D2	537	64	70	0·0022	Saline
D1	547	80	30	N.T.	Saline
D2	547	80	80	N.T.	Saline
D8	547	80	80	N.T.	Saline
D9	547	80	80	N.T.	Saline
D10	547	80	85	N.T.	Saline
D11	547	80	75	N.T.	Saline
D12	547	80	85	N.T.	Saline
D13	547	80	90	N.T.	Saline

(N.T. = not tested.)

It would appear that, from a recovery standpoint, the antitoxin D1, originally chosen for experimental work, was a very poor one indeed.

Two experiments were conducted to ascertain the purity obtained when refined antitoxin and unrefined antitoxic serum were used for flocculation purposes. The results are recorded in Table 5. Although the purity of the D.F. of toxoid 529

Table 5. *Purities using refined and unrefined serum*

Toxoid	Serum	D.F. (mg. N/Lf)
527/9	Refined	0·00208
	Unrefined	0·00206
529	Refined	0·0021
	Unrefined	0·0032

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prepared with unrefined serum was somewhat less than that made with refined antitoxin, it is questionable if this is significant. With toxoid 527/9, there was no significant difference in the purities of the D.F.'s.

A decided practical advantage would be obtained if a purer product and/or a larger recovery could be obtained by flocculating at a point either above or below the equivalence value, instead of at the equivalence value. In Table 6 the recoveries and purities obtained, using four different toxoids, are recorded. These

Table 6. *Recovery, purity, and amount of antitoxin used for flocculation*

Toxoid	Units A.T./Lf toxoid	D.F. Recovery (%)	D.F. mg. N/Lf
524	0·75	45	0·00237
	1·0	67	0·00176
	1·2	72	0·0019
425	0·67	50	0·0014
	1·0	50	0·00228
	2·0	31	0·00375
527/9	0·75	60	0·00221
	1·0	66	0·00208
	1·5	53	0·00311
529	0·75	50	0·0018
	1·0	50	0·0018
	1·4	50	0·0018

results show that there are no consistent differences either in the purities or the recoveries when flocculation is carried out at the equivalence value or above or below it.

Attempts to purify dissolved floccules

It can be assumed that dissolved floccules are made up of toxoid and of antitoxin, the latter so denatured, degraded, or altered that it is no longer capable of neutralizing toxin or toxoid. If this useless protein could be eliminated, a toxoid free, or almost free, from extraneous matter would be obtained. Using *Cl. welchii* dissolved floccules, a very considerable amount of work had been carried out with this end in view. It was found that any process that precipitated the toxoid, such as treatment with alcohol, sodium or ammonium sulphate, or lowering of the pH, also brought down the extraneous proteins. It is more likely that the protein of antitoxin origin, to which the toxoid was attached, was precipitated and carried the toxoid down with it. A considerable amount of extraneous material could sometimes be removed by heating the dissolved floccules at 93° C. for 5 min.; not infrequently a copious precipitate formed leaving a water-clear, much purer supernatant. However, the overall recovery was very low—of the order of 5–10 %.

Similar results were obtained with diphtheria-dissolved floccules, the reason becoming obvious when electrophoretic analysis, carried out by my colleague, C. G. Anderson, revealed only one component. Thus, it would appear that the toxoid portion of the complex is in very close association with the altered antitoxic part.

Finally, it may be said, that papaic, peptic and tryptic digestion destroyed the toxoid portion of the dissolved floccules.

Most of the nitrogen in D.F. comes from the altered antitoxin and there is no chemical means of knowing how much bacterial protein they contain, but by an immunological approach, it was shown that they contained no demonstrable amount. A substrain of the P.W.8 strain of *Corynebacterium diphtheriae* was cultivated on Linggood's and Fenton's medium containing 1 mg. of iron per 100 ml.; a good growth was obtained but the toxin content was < 2Lf/ml. filtrate, and the M.R.D. in a guinea-pig was 0·02 ml. Toxicity was completely removed by incubating the filtrate in the presence of 0·5 % formalin. Two rabbits were immunized by subcutaneous injections of this formolized material. Precipitation (ring) tests were put up, using the undiluted serum which contained 0·1 unit antitoxin/ml. and diluted antigens. The readings were made after the tubes had stood for 1 hr. at 37° C. and 1 hr. at 23° C. The results were as shown in Table 7. By this test no bacterial protein was demonstrable in D.F.

Table 7. *Precipitation (ring) tests*

		Antigen dilutions						
Antigen	Lf/ml.	1/1	1/2	1/4	1/8	1/16	1/32	1/64
'Iron' toxoid	< 2	+	+	+	+	±	−	−
Ordinary toxoid	60	+	+	±	±	−	−	−
D.F.	500	−	−	−	−	−	−	−

The antiserum was used undiluted.

Antigenicity of P.D.F. in guinea-pigs

A comparison of formol-toxoid (F.T.), A.P.T., T.A.F., D.F. and P.D.F.

Groups of guinea-pigs, ten per group, received 10 Lf of formol-toxoid 438, and 10 Lf of A.P.T., D.F. and P.D.F., all prepared from this toxoid. The antigens, diluted in saline to contain 10 Lf in 1·0 ml., were injected subcutaneously. T.A.F. was included, but as no flocculation value could be assigned to the undissolved floccule suspension, that volume corresponding to 10 Lf of dissolved floccules was given, again in a total volume of 1·0 ml. One month later, each animal was bled from the heart and, on the same day, received a further 10 Lf in 1 ml. of saline. After the lapse of another 14 days, the guinea-pigs were bled for a second time. The antitoxic content of the serum of each guinea-pig was titrated intradermally in the skin of normal guinea-pigs, using a well-matured and thoroughly tested toxin as indicator. The value of the test toxin was controlled in each guinea-pig by including an injection of the toxin, plus a known amount of International Standard antitoxin. The results are given in Table 8 as the geometric means of the serum titres of all the animals of each group.

There was such a poor response to one injection of toxoid, T.A.F. and D.F., that the geometric means could not be worked out. The responses to one injection of A.P.T. and P.D.F. were also poor, most probably caused, as will be shown later, by the small amount of precipitates injected, precipitates, moreover, that were

Table 8. *Geometric means of antitoxic titres of sera of guinea-pigs immunized with* 10 *Lf of toxoid, P.D.F., A.P.T., D.F. and with* 0·2 *ml. of T.A.F.*

G.M. = geometric mean; A.T. = antitoxin; (*a*) = actual titres; smallest amount of antitoxin tested for was 0·02 u./ml.

	G.M.'s (units A.T./ml.)	
Antigen	After one injection	After two injections
Toxoid	4/10: 0·02 u./ml., 6/10: <0·02 u./ml. (*a*)	2·9
P.D.F.	0·23	12·3
A.P.T.	0·19	9·7
D.F.	1/10: 0·1 u./ml., 9/10: <0·02 u./ml. (*a*)	1·4
T.A.F.	10/10 <0·02 u./ml. (*a*)	0·62

easily and quickly soluble in citrate solution (see p. 427). However, after two injections, the A.P.T. and the P.D.F. proved very good antigens, the response to P.D.F. being slightly better than that to A.P.T., and much better than that to T.A.F. and D.F., which, even after two injections, did not produce good immunity. The toxoid itself was mediocre as compared with the alum precipitates, although, if the titres of two unresponsive guinea-pigs are excluded, the result would have been very satisfactory (G.M. = 10 u./ml.). This experiment shows that P.D.F., as an antigen, is greatly superior to T.A.F. and D.F., is better than toxoid, and is as good as, if not better than, A.P.T.

The antigenicity of 2×1 *and* 2×10 *Lf of P.D.F.*

An objection could be advanced against drawing conclusions from the previous experiment in that a relatively large amount (10 Lf) of antigen was injected. It might be said that the large amount of antigen injected could mask real differences in antigenicity, because it is well known that two injections of a big dose of an indifferent antigen produce results not greatly inferior to two small doses of a good antigen. Differences can be shown up when the doses are reduced. In a new test, two groups of guinea-pigs were immunized with the same batch (425/38) of P.D.F., one lot receiving 2×1 Lf and the other lot, 2×10 Lf. The geometric means of the antitoxin titres 14 days after the second injection were respectively 13 and 17 u./ml. of serum. Thus, it is obvious that the response is not proportional to the amount of antigen injected.

Comparison of P.D.F. with antigens prepared by other laboratories

Through the courtesy of Mr L. B. Holt of St Mary's Hospital, a sample of dry, purified toxoid, and of purified toxoid adsorbed on aluminium phosphate, as issued by him, were received. In addition, the Director of the Wellcome Research Laboratories was good enough to place formol-toxoid, A.P.T., and T.A.F. at my disposal. From the State Serum Institute, Copenhagen, through the kindness of Dr Inga Scheibel, a sample of purified toxoid, adsorbed on $Al(OH)_3$, was received.

The dry toxoid was dissolved in a small volume of saline and added to a suspension of $AlPO_4$ containing 7·5 mg. $AlPO_4$/ml. to give 16 Lf/ml. Holt's preparations were diluted to the required Lf strength in $AlPO_4$ suspension (7·5 mg./ml.)

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following the method described by him (Holt, 1947); the other antigens were diluted in saline except the T.A.F. which was used undiluted. Table 9 records the results, as geometric means of the antitoxic titres per ml. of serum of guinea-pigs immunized by one and two injections.

Table 9. *P.D.F. compared with other antigens*

D. = Danish; W. = Wellcome; H. = Holt; N.T. = not tested; (*a*) = actual values, units/ml. serum.

Antigen	Dose injected			Geometric means (units A.T./ml.) after	
	Lf	ml.	Diluent	One injection	Two injections
P.D.F. 477	2	1	Saline	0·3	11·1
D. $Al(OH)_3$	1	1	Saline	0·05	16·0
W. toxoid	2	1	Saline	1/10: 0·02 u., 9/10: < 0·01 u. (*a*)	0·39
W. A.P.T.	2	1	Saline	0·3	12·7
W. T.A.F.	—	0·2	—	N.T.	0·74
H. dry	1	0·5	$AlPO_4$	1·5	15·0
H. dry	0·5	0·3	$AlPO_4$	N.T.	4·0
H. dry	5	0·33	$AlPO_4$	N.T.	8·0
H. issue	3·3	1	$AlPO_4$	2·45	9·1

It is clear that Holt's antigen, in $AlPO_4$, provided the best primary stimulus, 1 Lf in 0·5 ml. $AlPO_4$, giving rise to 1·5 u./ml. and 3·3 Lf in 1 ml. $AlPO_4$ to 2·45 u./ml. As will be shown later, this was not necessarily due entirely to an inherent superiority of the antigen as antigen but rather to the larger amount of carrier, $AlPO_4$, injected with it. The Wellcome liquid toxoid and the Danish prophylactic provided little immunity after one injection and, from the poor response, 0·74 u./ml., to two injections of Wellcome T.A.F., it can be assumed the immunity produced by the primary stimulus to that preparation was poor also. One injection of both P.D.F. and Wellcome A.P.T. in saline stimulated the formation of 0·3 u./ml., considerably inferior to that resulting from the injection of Holt's antigen in $AlPO_4$.

The picture was very different after the secondary stimulus, the effect of the large amount of carrier, so apparent after only one injection, being completely nullified.

The differences in the titres produced by the various antigens after one injection is, in great measure, to be ascribed to the amount of carrier injected and not to the antigen as such. This point is brought out in the following experiments.

The following antigens were prepared:

(1) D.F. 494, of 440 Lf/ml., was adsorbed on $AlPO_4$ (7·5 mg./ml.) so that 1 ml. contained 50 Lf; this was further diluted in $AlPO_4$ or in saline, so that the dose to be injected contained 2 Lf.

(2) P.D.F. was made from D.F. 494, using 1 % alum, and diluted in saline to contain 2 Lf per dose.

(3) D.F. 516, of 350 Lf/ml., was adsorbed on $AlPO_4$ so that 1 ml. contained 50 Lf; this was further diluted in $AlPO_4$ or in saline so as to contain 1 Lf per dose.

(4) P.D.F. was made from D.F. 516 of 350 Lf/ml., in the following manner. The concentrated D.F. was diluted in saline to contain 60 Lf/ml., and alum solution added to give a 1 % concentration. The pH, in this instance, was adjusted to 8·3 and not, as previously, until precipitation occurred at between pH 5·5 and 7. The precipitate produced at the higher pH was, after washing with saline, only slowly and not completely soluble in citrate solution; the insoluble material was probably $Al(OH)_3$. This P.D.F. was diluted in $AlPO_4$ or in saline so that the dose to be injected contained 1 Lf.

Guinea-pigs (ten per group) were immunized and their sera tested for antitoxin after one and two injections. Table 10 summarizes the results.

Table 10. *Geometric means of antitoxin titres of sera of guinea-pigs immunized with D.F. or P.D.F. undiluted, or diluted in saline or in* $AlPO_4$

Antigen	Diluent	Vol. (ml.)	Lf	Geometric means (unit A.T./ml.) After one injection	Geometric means (unit A.T./ml.) After two injections
(1) D.F. 494 $AlPO_4$ ads.	Saline	0·5	2	0·37	14·6
(2) D.F. 494 $AlPO_4$ ads.	$AlPO_4$	0·5	2	1·3	9·8
(3) P.D.F. 494 1 % alum	Saline	1·0	2	0·11	9·7
(4) D.F. 516 $AlPO_4$ ads.	Saline	1·0	1	0·2	18·6
(5) D.F. 516 $AlPO_4$ ads.	$AlPO_4$	0·5	1	1·0	11·3
(6) D.F. 516 $AlPO_4$ ads.	Undiluted	0·2	10	1·5	11·0
(7) P.D.F. 516 1 % alum,* pH 8·3	Saline	1·0	1	0·15	9·5
(8) P.D.F. 516 1 % alum, pH 8·3	$AlPO_4$	0·5	1	1·7	12·3
(9) P.D.F. 516 1 % alum, pH 8·3	Saline† and $AlPO_4$	1·0	1	0·43	15·8
(10) P.D.F. 516 1 % alum, pH 8·3	Undiluted	0·2	10	2·8	20·9

* See text.

† Diluted in saline, with enough $AlPO_4$ added to give the same opacity as (4).

Ads. = adsorbed; vol. = volume.

The effect of the large amount of carrier on the immunity produced after only one injection is clearly brought out. The results in Table 10 show that 2 Lf of D.F. 494 in 0·5 ml. of $AlPO_4$, no. (2), gave rise to a geometric mean of 1·3 u./ml. against 0·37 u./ml. when saline no. (1) was the diluent. Differences of the same order were obtained with D.F. 516, nos. (4) and (5), and P.D.F. nos. (7) and (8). Antigen no. (9), which was the same as no. (7) except that the opacity was increased by the addition of $AlPO_4$ to correspond to that of antigen no. (4), produced 0·43 u./ml. as against 0·15 u./ml. for no. (7).

The differences in titre after two stimuli were not nearly so marked, the effect of the carrier being annulled.

Antigens nos. (6) and (10) were introduced further to test the carrier question. These were antigens of the carrier and Lf strength that would be injected into man. The method laid down in the British Therapeutic Substances Regulations for the testing of A.P.T. does not seem fair to the product. An antigen, A.P.T., that will be slowly absorbed when injected into man in a dose of from 0·2 to 0·5 ml. (10–25 Lf) is, in the guinea-pig test, diluted to 1 Lf in 1 ml. saline, thus removing, in great measure, the property, slow absorbability, that was the main reason for preparing it. Holt (1947) overcame this difficulty by diluting his antigen in $AlPO_4$ of the same carrier strength as used in man. This procedure, as has been noted, can be used for P.D.F., but becomes something of an immunological 'trick' in that the alum precipitate, and not the precipitate mixed with $AlPO_4$, is used for immunizing man. In general, it can be said that two small, spaced, injections of an antigen produce a better immunity than one large dose. Further, it has been shown (p. 425) that the number of Lf doses of diphtheria toxoid is not clearly correlated with the immunity produced. If one dose of P.D.F., containing sufficient precipitate and/or carrier to form a small, slowly absorbable depot, could immunize guinea-pigs solidly, it was probable that two larger doses would immunize man.

The results in Table 10 show that 0·2 ml. of P.D.F. 516 of Lf 50/ml., no. (10), and 0·2 ml. of D.F. 516 of Lf 50/ml. adsorbed on $AlPO_4$, no. (6), were good antigens, no. (10) being almost twice as good as no. (6) after both one and two injections.

The question of the fairest method of testing the intrinsic value of a diphtheria antigen arises. It is obvious, from the results recorded, that, depending on the amount of carrier used and on its absorbability, and on whether one or two stimuli are given, widely varying results can be expected.

The most striking example was that using the Danish prophylactic, noted in Table 9, when the primary stimulus produced only 0·05 u./ml. and the secondary stimulus 16 u./ml. For A.P.T. and P.D.F., the method used for testing antigen no. (10) in Table 10 is probably very good and, for $AlPO_4$-adsorbed antigens, Holt's method of injecting 1 or 2 Lf in 0·5 ml. of $AlPO_4$ is practically sound, but it is doubtful if either of these methods would show up a difference in two antigens, both good, but one twice as good as the other. Probably 1 Lf is a large amount of antigen for a guinea-pig and provides a sufficiently large stimulus to mask true differences in antigenicity. There was the possibility that one or two stimuli of a very small dose of antigen, 0·1 Lf, absorbed on, or suspended in, 0·5 ml. $AlPO_4$ would reveal differences. This method again calls in the aid of a large dose of carrier, but this is unavoidable, as P.D.F., A.P.T., or $AlPO_4$-adsorbed toxoids so diluted in saline to contain 0·1 Lf could not be expected to provide much immunity after two stimuli, let alone after one stimulus.

To check this assumption, P.D.F. 516, A.D.F. 516, A.P.T. 78, A.D.F. (1) and Holt's $AlPO_4$-adsorbed toxoid were diluted in $AlPO_4$ (10 mg./ml.). Guinea-pigs were immunized with 0·1 Lf in 0·5 ml. of $AlPO_4$ and their sera tested, after one and two injections, for antitoxin content. The results are recorded in Table 11.

Table 11. *Antigenicity in guinea-pigs of* 0·1 *Lf of P.D.F.*516, *A.D.F.*516, *A.D.F.* (1), *Holt's P.T.A.P. and A.P.T.*78 *in* 0·5 *ml. of* $AlPO_4$ (10 *mg./ml.*)

	P.D.F. 516 (units A.T./ml. serum after injection)		A.D.F. 516 (units A.T./ml. serum after injection)		A.D.F. (1) (units A.T./ml. serum after injection)	
G.P.	1	2	1	2	1	2
1	0·04–0·1	3	0·01–0·02	1–1·5	0·2–0·5	3 –5
2	0·1 –0·2	2	0·01–0·02	0·75–1	0·2–0·5	3 –5
3	0·2 –0·5	7–10	0·04–0·1	3–5	0·2–0·5	3 –5
4	0·2 –0·5	5–7	0·1 –0·2	5	0·2–0·5	3 –5
5	0·2 –0·5	5–7	0·1 –0·2	3–5	0·2–0·5	1·5–2
6	0·5 –0·75	7–10	0·2	5	0·5	3 –5
7	0·75	5	0·2	2–3	0·5	7 –10
8	1–1·5	—	0·2 –0·5	3	1 –1·5	5 –7
9	1–1·5	—	0·2 –0·5	7	1·5	10
10	—	—	1	5–7	—	—
G.M.	0·39	4·96	0·13	3·2	0·48	4·49

	P.T.A.P. (units A.T./ml. serum after injection)		A.P.T. 78 (units A.T./ml. serum after injection)	
G.P.	1	2	1	2
1	0·01	0·75–1	?0·0012	0·2–0·5
2	0·04	2–3	?0·0025	0·75
3	0·1	3	<0·005	0·5–0·75
4	0·1	3	<0·005	2
5	0·1	3	0·01–0·02	1·5
6	0·2	5	0·04–0·1	2
7	0·2	5	0·1 –0·2	5
8	0·5	3	0·2 –0·5	5–7
9	0·75	3	0·2 –0·5	5
10	—	—	—	—
G.M.	0·12	2·86	—	1·71

G.M. = geometric mean.

Statistically, after both one and two injections, P.D.F.516 and A.D.F.(1), as antigens, are not significantly different from one another but are significantly better than A.D.F.516, P.T.A.P. and A.P.T. However, except for the A.P.T., no great importance is attached to this, because the presence of a few unresponsive guinea-pigs in one group and that of a few very responsive animals in another group lowers or raises the geometric mean very considerably. Further, experiments, not recorded here, indicate that an undue proportion of unresponsive guinea-pigs was included in the A.P.T. group. However, the results summarized in Table 11 do show that P.D.F. and A.D.F. are good antigens.

The optimum amount of carrier is at present being investigated, and preliminary results with A.D.F. indicate that 0·5 mg. $AlPO_4$/0·1 Lf gives slightly better results than 5 mg. $AlPO_4$/0·1 Lf and with P.T.A.P. slightly worse results.

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DISCUSSION AND SUMMARY

Since the introduction of toxoid-antitoxin floccules by Glenny & Pope (1927) little has been done to improve their value as an antigen. Watson, Taggart & Shaw (1941) and Barr (1949) confirmed the work of these investigators in showing that heating at 80° C. increased their antigenic value.

It has been shown in this article that about 80 % of the toxoid contained in floccules may be recovered if they are dissolved in N/20-NaOH, the alkali being allowed to act for 1 hr. before being neutralized with acid. A purity of between 0·002 and 0·0025 mg. N/Lf is obtained regularly. Such a dissolved floccule solution is a relatively poor antigen when tested in guinea-pigs, but may be rendered highly antigenic when precipitated with alum or adsorbed on $AlPO_4$.

The method has the advantage of simplicity. It would appear that any medium capable of yielding a toxin convertible into flocculable toxoid may be used, thus doing away with cumbersome media-making methods. However, for the best results in terms of both recovery and purity, a high-value, quickly flocculating, toxoid should be used. Further, the choice of antitoxin appears to be important where recovery is concerned. With most of the antitoxins used, recoveries of between 70 and 90 % were got, although with that used for most of the work recorded here, the recoveries were much lower.

It is not yet established which is the antigen of choice—alum-precipitated (P.D.F.) or $AlPO_4$-adsorbed (A.D.F.) dissolved floccules. A.D.F. has the advantage of ease of preparation and of constant mineral carrier content, but further work is necessary to establish the optimum amount of carrier before a definite answer can be given. A paper on immunity responses in man will be published shortly in the *Lancet*.

I have pleasure in recording my indebtedness to my colleagues, C. G. Anderson and D. S. du Toit, for the many nitrogen estimations they carried out.

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